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Development of a real-time PCR for the identification of *Bordetella pertussis* and *Bordetella parapertussis*

A. Ménard[1], P. Lehours[1], J. Sarlangue[2], C. Bébéar[1], F. Mégraud[1] and B. de Barbeyrac[1]

[1]Laboratoire de Bactériologie and [2]Hôpital Pédiatrique, CHU Pellegrin, Bordeaux, France

ABSTRACT

This study describes a real-time PCR assay for the detection and identification of *Bordetella pertussis* and *Bordetella parapertussis*. The assay is based on amplification of a fragment from the repeat sequence regions IS*481* and IS*1001* found in *B. pertussis* and *B. parapertussis*, respectively, with subsequent species identification by melting curve analysis using SYBR Green chemistry. Discrimination between the two species was straightforward, as the corresponding melting points showed a significant difference of 7°C. The assay was evaluated first with reference strains and retrospective human clinical samples, and then prospectively with 132 human clinical specimens received between April 2003 and December 2005. The assay allowed the rapid detection of 22 positive clinical samples, of which 15, including one fatal case, were not identified by standard culture techniques. The new assay was sensitive and specific, and can be implemented easily using any real-time PCR apparatus.

Keywords *Bordetella*, diagnosis, identification, pertussis, real-time PCR

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INTRODUCTION

A resurgence of pertussis infection has been observed in France, with an increase to 642 clinical cases during the year 2000, of which nine were fatal [1]. The number of infants hospitalised has increased, and the presence of whooping cough syndrome among other individuals present in their environment has been noted. This disease now affects young adults and teenagers who were vaccinated >10 years previously, thus enabling them to contaminate unvaccinated or incompletely vaccinated children, who are thereby exposed to severe forms of the disease. In this adult and teenage population, the pertussis infection evolves readily into an atypical form that is difficult to diagnose, so that microbiological confirmation is necessary.

Corresponding author and reprint requests: A Ménard, INSERM ERI 10, Laboratoire de Bactériologie, Zone Nord, Bâtiment 2B, Université Victor Segalen Bordeaux 2, 146 Rue Léo Saignat, 33076 Bordeaux cedex, France
E-mail: armelle.menard@labhel.u-bordeaux2.fr

Molecular biological techniques provide invaluable tools for the diagnosis of *Bordetella pertussis* infection, and the use of PCR in reaching a diagnosis has been recommended for several years [2]. PCR is more sensitive than culture for the detection of *B. pertussis* and *Bordetella parapertussis*, especially during the late stages of the disease and/or after antibiotic treatment has been started [3]. Since 2001, numerous real-time PCR techniques have been developed for the detection and identification of *B. pertussis*. Most of these assays target the IS*481* sequence [4–11], the toxin gene [12,13] or the pertactin gene [14], and use one of the three real-time detection formats currently available, i.e., SYBR Green, *Taq*Man or fluorescence resonance energy transfer chemistry. However, only two of the published assays used SYBR Green chemistry and melting curve analysis (MCA), amplifying the IS*481* sequence and the toxin promoter region, respectively [11,15]. Following the resurgence of whooping cough in France, the present study describes the development of a real-time PCR assay using SYBR Green chemistry with the repeated IS*481* sequences of *B. pertussis* and the repeated IS*1001* sequences of

B. parapertussis as targets. Subsequent species identification was based on MCA. The advantages of this new test are the relatively low cost (since no probe is used) and the possibility of identifying both of the above species in a single test.

MATERIALS AND METHODS

Bacterial strains

Several strains of *Bordetella* spp., including *B. pertussis* Sato (CIP81.32), *B. parapertussis* 22651 L2 (CIP63.2), *Bordetella bronchiseptica* type strain 452 (CIP55.110) and *Bordetella holmesii* type strain 5589 (CIP 104394), were used in this study and were cultured on selective media as described below. Other bacterial species isolated from clinical specimens and used to determine the specificity of the PCR primers included *Escherichia coli*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Neisseria meningitidis*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. Genomic DNA was extracted using the MagnaPure LC DNA Isolation Kit I and the MagnaPure LC Isolation Station (Roche Applied Science, Penzberg, Germany). DNA was stored at −20°C until required for analysis.

Nasopharyngeal aspirates

Twenty-nine frozen nasopharyngeal aspirates (NPAs), collected originally at the Children's Hospital of Bordeaux between June 1996 and March 2003 (male/female ratio, 0.52; mean age, 3.4 ± 3.3 months), were used for the retrospective study, of which 17 were culture- and direct fluorescent antibody test-positive, and 12 were culture- and direct fluorescent antibody test-negative.

For the prospective study, 132 NPAs were used. Three samples were received from private laboratories (male/female ratio, 0.33; mean age, 31.2 ± 26.1 years) and 129 were obtained from neonates and infants between April 2003 and December 2005 (male/female ratio, 0.51; mean age, 2.6 ± 7.2 years). The time between NPA collection and laboratory analysis was < 15 min. Once received, each NPA was used to inoculate two selective media, Reagan Lowe medium (charcoal agar; Oxoid, Basingstoke, UK) and Bordet Gengou medium (Bordet Gengou agar base; Difco, Detroit, MI, USA), supplemented with defibrinated sheep blood 5% v/v. The plates were incubated at 37°C and examined for 5 days for colonies typical of *B. pertussis* or *B. parapertussis*. Representative colonies with typical Gram-negative coccobacilli morphology were confirmed by direct fluorescent anti-*B. pertussis* antibody tests (Bacto FA *B. pertussis*; Difco) and the API 20E system (bioMérieux, Marne-la-Coquette, France) using a MacFarland × 4 suspension. Each isolate was sent to the French National Reference Centre for *Bordetella* (Pasteur Institute, Paris, France) for confirmation of species identification.

Genomic DNA was also extracted directly from each NPA with the MagnaPure LC DNA Isolation Kit I and the MagnaPure LC Isolation Station. DNA was stored at −20°C until required for analysis. Extracted DNA was tested both undiluted and diluted 1:10 using the three PCRs described below.

Table 1. PCR primers used in this study

Primers	Sequences (5′ → 3′)	Target	Size (bp)	T_m (°C)
F1-BP	TCCGAACCGGATTTGAGAAA	IS*481*	60	78.5
R1-BP	CCGGGCTCCTTGAGTGAA			
F1-BpP	ATGCTGGATCGCAAGTTGATG	IS*1001*	81	85.8
R2-BpP	TGGGTCTTCGGGCCATT			
F1-16S-TQM	CGTGTCGTGAGATGTTGGGTTA	16S rDNA	60	78.5
R1-16S-TQM	GACGTCATCCCCACCTTCCT			

Design of the primers for PCR amplification of the IS*481*, IS*1001* and 16S rDNA sequences

The available IS*481* sequences of *B. pertussis* (GenBank accession numbers M28220, 66937, L26973, S66929 and M22031) were aligned using multiple sequence alignment with hierarchical clustering [16] (http://prodes.toulouse.inra.fr/multalin/multalin.html) and analysed to identify conserved regions for the primer design. Primers for the detection of *B. parapertussis* were designed from the available IS*1001* sequence (GenBank accession number X66858).

Seventy 16S rDNA sequences from diverse bacterial species found commonly in humans, including *Moraxella catarrhalis*, *Chlamydia pneumoniae*, *N. meningitidis*, *H. influenzae*, *H. parainfluenzae*, *B. pertussis*, *B. parapertussis*, *B. holmesii*, *B. bronchiseptica*, *E. coli*, *Salm.* Enteritidis, *Salm.* Typhimurium, *M. pneumoniae*, *M. genitalium*, *Strep. pneumoniae* and *Staph. aureus*, were aligned and primers were designed to target a conserved region. The resulting three primer sets (Table 1) were designed using the Primer Express software package (Applied Biosystems, Foster City, CA, USA) and were synthesised by Eurogentec (Seraing, Belgium).

Real-time PCR and MCA analysis

SYBR Green real-time PCR amplification and MCA were carried out in a final volume of 25 μL containing 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems), 0.3 μM each primer and 5 μL of purified DNA in an ABI PRISM 7000 thermocycler (Applied Biosystems). Amplification parameters comprised 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. This was followed by the melting program of the ABI PRISM 7000 thermocycler, with continuous monitoring of the fluorescence (dissociation program).

RESULTS AND DISCUSSION

Development of the assay

The IS*481* sequences were chosen because PCRs targeting these repeated sequences are more sensitive than PCRs targeting the toxin promoter [3]. The IS*481* primers were designed in order to avoid primer dimers that could interfere with the interpretation of the results. A universal PCR targeting the 16S rRNA gene was also developed to be used as a control to monitor the extraction and absence of PCR inhibitors. The PCR primer sets were tested on DNA extracted from the

Bordetella spp. and other bacterial species listed above. As expected, DNA extracted from all of the strains yielded PCR products with the F1/R1-16S-TQM primer set targeting 16S rRNA genes. Only DNA extracted from *B. pertussis* yielded amplification products with the F1/R1-BP primer set, and only DNA extracted from *B. parapertussis* yielded amplification products with the F1/R2-BpP primer set. MCA of the 60-bp and 81-bp amplicons from the *B. pertussis* and *B. parapertussis* control strains showed melting temperatures (T_m) of *c.* 79°C and 86°C, respectively.

Retrospective study

A retrospective study was performed on culture- and direct fluorescent antibody test-positive NPAs (n = 17) and on culture- and direct fluorescent antibody test-negative NPAs (n = 12), all of which were tested undiluted and diluted 1:10. The expected peak corresponding to *B. pertussis* was generated for the 17 positive samples, with no peak for the 12 negative samples. No additional peak was ever observed with these two PCRs, including all of the dilutions tested, or the specimens tested (water, control strains and NPA). Even after 40 PCR cycles, no primer dimers were observed (data not shown).

Sensitivity of the test

The sensitivity of the PCR for the detection of *B. pertussis* and *B. parapertussis* was evaluated using ten-fold serial dilutions of a bacterial suspension in phosphate-buffered saline, which was quantified by culture on specific media and subsequent colony counts. PCR with the DNA extracted from the bacterial suspensions yielded regression curves with slopes of −3.379 and −3.323 for *B. pertussis* and *B. parapertussis*, respectively, i.e., very close to a slope of −3.32, which would correspond to the maximum efficiency. For the two PCRs, the regression curve was linear from 10^5 to one bacterial genome with a reaction volume of 25 μL.

Prospective study

In total, 132 successive clinical specimens received at the Hospital of Bordeaux between April 2003 and December 2005 were tested, and the PCR results were compared with the results of culture. All of the NPAs gave a positive result with the 16S rDNA PCR, thereby demonstrating that DNA extraction from each specimen had been successful and that the PCR was not inhibited. Of the 132 clinical samples tested, seven were positive by culture (one *B. bronchiseptica* and six *B. pertussis*), while 22 were positive by PCR for *Bordetella* spp. (three *B. parapertussis* and 19 *B. pertussis*), i.e., three times as many positive results by PCR than by culture. The identification results are summarised in Table 2.

Table 2. Culture and PCR identification results for *Bordetella* spp. among 132 nasopharyngeal aspirate samples included in the prospective study

Number of patients	Culture-positive	PCR-positive
110	Negative	Negative
3	Negative	*B. parapertussis*
12[a]	Negative	*B. pertussis*
6[b]	*B. pertussis*	*B. pertussis*
1[c]	*B. bronchiseptica*	*B. pertussis*
Total = 132	(7 positive)	(22 positive)

[a,b]These groups contain one and two nasopharyngeal aspirate samples, respectively, taken from adults.
[c]Confirmed as *B. bronchiseptica* by 16S rDNA sequencing and by the French National Reference Centre for Bordetella, although the PCR (on nasopharyngeal aspirates and on the purified isolate) was positive for *B. pertussis.*

The results for the seven cases positive by both culture and PCR were in agreement, with the exception of one isolate of *B. bronchiseptica* that was identified by culture, but was identified erroneously as *B. pertussis* by PCR. An identical PCR result was obtained with genomic DNA extracted from the purified isolate, thereby confirming the presence of IS*481* sequences from *B. pertussis* in this *B. bronchiseptica* strain, as reported previously [17]. The young infant infected by *B. bronchiseptica* exhibited clinical symptoms of whooping cough, but cases of whooping cough associated with *B. bronchiseptica* infection are rare and are often associated with underlying conditions (e.g., immunosuppression) [18] or are acquired by contact with pets vaccinated orally with attenuated strains [19]. This result indicates a continued need to maintain cultural techniques in order to follow the epidemiology of *B. bronchiseptica* infections, and an additional need to monitor the specificity of IS*481* sequences [3] or to use another target gene, e.g., the toxin promoter region.

Among the 22 PCR-positive cases, there were three adult women (mean age, 53.6 ± 25.3 years) and 19 children (male/female ratio, 0.58; mean age, 4.8 ± 6.6 months), of whom 82.3% were aged <6 months. Of the 19 children, 70.6% had not been vaccinated, and 17.6% had received only

one dose of vaccine; the vaccination status was unknown for 11.8% cases. These children were admitted to the hospital for a progressive cough, which began <8 days before admission in 17.7% of cases and ≥8 days before admission in 82.3% of cases. The cough continued to evolve over >21 days in 58.8% of these cases. Among the more serious symptoms, 29% of the children suffered from periods of apnoea, and 17.7% of the children were admitted to the intensive care unit. A hyper-lymphocytosis was detected in 29.4% of cases, and a respiratory syncytial virus co-infection was detected in one case. All of the children were treated with macrolides; overall, the clinical outcome was positive, but one infant died. In 41.2% of the cases, a contaminant organism (either suspected or proven) was identified and, furthermore, was found in 85.7% of the families of these children.

The infant who died was unvaccinated and was aged 2.5 months. He was admitted to the paediatric intensive care unit in June 2005 for respiratory distress, with a 3-day history of a progressive cough, despite treatment with josamycin. Severe hypoxaemia and significant pulmonary arterial hypertension were observed upon admission. Initial laboratory tests revealed a white blood cell count of 118 Giga/L (66% segmented neutrophils and 26% lymphocytes) and a platelet count of 768 Giga/L. A chest X-ray showed bilateral and complete densities in the lungs. An NPA was negative for pertussis by culture, but the PCR was positive for *B. pertussis*, which supported the diagnosis of malignant whooping cough and eliminated the possibility of haematological disease. NPAs from the parents were negative by culture, but the NPA from the mother, aged 23 years, was positive by PCR. The mother had complained of a simple cough 15 days earlier. Despite assisted ventilation, the child died 17 days after admission to the paediatric intensive care unit.

Although this malignant form of pertussis remains very rare, it is always a severe disease in infancy. This case illustrates: (i) the importance of PCR in the diagnosis of whooping cough, considering the limitations of culture [3], especially in atypical cases and after initiation of an antibiotic treatment; (ii) the need to identify carriers in order to avoid further infections in the community and to establish an epidemiological link; and (iii) the importance of the recent changes in the French immunisation schedule, including the recommendation of a pertussis vaccine booster for young adults, particularly at the time of pregnancy [20].

Nucleic acid amplification tests are now increasingly relevant for the detection and identification of *B. pertussis* and *B. parapertussis*, particularly in view of the lack of sensitivity of culture for detecting these bacteria. Three real-time PCR detection formats are available (SYBR Green, *Taq*Man or fluorescence resonance energy transfer chemistries). The SYBR Green format has the advantage of being implemented easily on different real-time PCR machines. Two PCRs using SYBR Green and MCA of the resulting amplicons have been described previously for detection of *B. pertussis* and *B. parapertussis*. The first of these, targeting the promoter of the toxin subunit S1 of *B. pertussis* and the toxin pseudogene of *B. parapertussis* [15], generated PCR products with T_ms of 89.8°C and 91.7°C, respectively, but also generated non-specific products with a T_m of *c.* 81°C, probably corresponding to double-stranded primer dimers, that interfered with the interpretation and/or quantification. Moreover, this PCR was less sensitive than a second PCR targeting the IS*481* and IS*1001* repeated sequences of *B. pertussis* and *B. parapertussis* [3,11], although the latter PCR also generated non-specific products. The PCR described in the present study also targets the IS elements of these bacteria, but no non-specific peaks were ever detected, and the sensitivity level was comparable to those reported previously using either SYBR Green and MCA [11], a fluorescence resonance energy transfer probe [7] or a *Taq*Man probe [8]. It should be noted that the presence of repeated IS elements means that PCRs specifically targeting such elements are often more sensitive, but the fact that these sequences are also transposable elements [21] may reduce the specificity. Indeed, a fragment from IS*481* has been detected in *B. holmesii* [22] as well as in *B. bronchiseptica* (this study). These observations suggest that complementary PCRs targeting a specific gene, e.g., the toxin or the pertactin genes [12–14], may be useful in providing support for interpretation of the assay result.

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